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Gender determination in parrots from Korean zoos using chromo-helicase-DNA binding protein 1 (*CHD*1) gene fragments

Jung-il Kim, Thinh Dinh Do, Tae-June Choi, Yonggu Yeo[1] and Chang-Bae Kim*

Department of Biotechnology, Sangmyung University, Seoul 03016, Republic of Korea
[1]Conservation and Health Center, Seoul Zoo, Gwacheon 13829, Republic of Korea

***Corresponding author**
Tel. 02-2287-5288
Fax: 02-2287-0070
E-mail. evodevo@smu.ac.kr

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Abstract: Many parrots are considered endangered species due to threats from human activities. Gender determination is of great importance for biological studies and the conservation of endangered parrots. However, like other birds, gender determination in parrots is hindered due to the lack of external dimorphism between males and females. A molecular approach using the chromo-helicase-DNA binding protein 1 (*CHD*1) gene is commonly used for sexing birds. This study aimed to determine the gender of parrots from Korean zoos based on amplification and visualization of the partial *CHD*1 gene. The samples of 13 parrot species were collected from three different zoos in Korea and the extracted DNA templates were amplified using *CHD*1 gene primers. The gender of 27 samples of 13 species was determined by visualizing the PCR products on an agarose gel. While male parrots were indicated by a single band, female parrots were indicated by double bands. The findings provide additional information, which might be helpful for the management and care of parrots in Korean zoos.

Keywords: parrot, molecular sexing, *CHD*1 gene, P8/P2 primers, gel electrophoresis

INTRODUCTION

It is a challenging task to determine gender in many avian species based on external morphology. Griffits and colleagues reported that more than 50% of bird species are monomorphic (Griffits *et al.* 1998). Moreover, gender determination is difficult in the juvenile stage, even for dimorphic species (Kahn *et al.* 1998). The difficulty in gender determination of bird is a hurdle for research, wildlife conservation, and breeding program. Gender determination is necessary for various avian conservation programs that target the protection of numerous species through intensive breeding (Ito *et al.* 2003). In zoo and breeding centers, the accuracy of gender determination is essential since a massive number of birds are bred, produced, and traded (Vucicevic *et al.* 2013). Also, gender determination plays a vital role in veterinary science, enhancing the protection and conservation of birds (Lee *et al.* 2010).

Due to the high demand in the market, parrots (order Psittaciformes) are one of the most traded among all avian orders (Bush *et al.* 2014). The human demand results in legal and illegal trade of parrots. Understanding of parrot gender will be helpful to breed and protect parrot from natural and human threats such as uncontrolled trade. However, like many other birds, parrots do not show a high degree of sexual dimorphism, even in the mature stage. In this case, applying molecular techniques is necessary for fast and accurate identification of gender in parrots.

In avian species, the male has homogametic sex chromosomes (ZZ), whereas the female has heterogametic sex

chromosomes (ZW). The presence of a chromo-helicase-DNA binding protein 1 (*CHD*1) gene on avian Z and W chromosomes with different size has increased the ability to determine gender for avian species (Griffiths and Tiwari 1995). The *CHD*1 gene is reported to encode the protein that regulates transcriptional activation on the chromatin level (Ellegren 1996). In chicken (*Gallus gallus*), *CHD*1-Z (gene ID: 395783) and *CHD*1-W (gene ID: 374195) were 48,421 bp and 115,681 bp in size, respectively and each gene includes 37 exons. Even though *CHD*1 genes encoded on Z and W chromosomes are different in length, the comparison of nucleotide sequences indicated that they are highly conserved (Valadan *et al.* 2017). For gender determination, based on *CHD*1 gene sequences of chicken and other animals, different universal primers were designed to flank *CHD*1 gene fragment with the intron (Griffits *et al.* 1998; Kahn *et al.* 1998). The amplified products contain the intron that is different in size between Z and W chromosomes. In chicken, P8/P2 primers bind to exons 23 and 24 and cover the intron between them. PCR amplification for partial *CHD*1-Z and *CHD*1-W fragments with P8/P2 primers is a quick and straightforward approach to determine avian gender (Griffits *et al.* 1998; Jensen *et al.* 2003). Avian gender is determined according to the observation of gel band where the sizes of the W and Z amplicons are differentiated.

In this study, the blind test to determine the gender of parrots from three Korean zoos was performed based on the amplification of the *CHD*1 gene. The primer P8/P2 (Griffiths *et al.* 1998) was used to amplify *CHD*1 gene fragments from different genders. The PCR products were visualized under UV light to determine the gender of collected parrot samples.

MATERIALS AND METHODS

A set of unknown gender samples of feather and blood examined in this study were obtained from parrots grown in Seoul Zoo (Seoul), Cheongju Zoo (Cheongju) and Uchi Zoo (Gwangju), Korea (Table 1). The ethical clearance number 2019-001 for the study using blood was issued by Seoul Zoo IACUC. Before the study started, all samples were confirmed species names based on cytochrome *b* gene sequences (data not shown). DNA was isolated from samples by Qiagen DNeasy® Blood & Tissue Kit (Qiagen Inc., Valencia, CA), following the manufacturer's instruction. The extracted DNA purity and concentration were measured by MaestroNano spectrophotometer (Maestrogen, Hsinchu, Taiwan).

Partial *CHD*1 gene was amplified with forward primer P8 (5′-CTCCCAAGGATGAGRAAYTG-3′) and reverse primer P2 (5′-TCTGCATCGCTAAATCCTTT-3′) described by Griffiths *et al.* (1998). PCR reaction mixture contained 30 μL: 15 μL of 2X DyeMix (Enzynomics, Daejeon, Korea) with 1.0 U of Taq polymerase, 1 μL of each primer (5 pmole μL^{-1}), 3 μL of DNA and distilled water up to 30 μL. The PCR condition: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 45 s, annealing at 52°C for 30 s, extension at 72°C for 30 s; followed by a final extension at 72°C for 5 min. After staining 5% agarose gel (w/v) with Midori Green Advance (NIPPON Genetics Europe, Dueren, Germany), PCR products were run in 90 minutes at 120 V and visualized under UV light to check there were one or two bands according to parrot gender. For estimation of *CHD*1 gene fragment size, 100 bp step DNA ladder (Bionics, Korea) was run together with the PCR products. For confirmation of *CHD*1 gene amplification, PCR products of monk parakeet (*Myiopsitta monachus*) were cloned by TOPcloner™ TA-Blunt kit (Enzynomics, Daejeon, Korea) and sequenced by Sanger sequencing. To check whether PCR products were *CHD*1 gene, the PCR products of monk parakeet (*Myiopsitta monachus*) were sequenced and submitted to Genbank (accession numbers: MT316044 for *CHD*1-Z and MT316045 for *CHD*1-W).

RESULTS AND DISCUSSION

*CHD*1 gene fragments from 27 parrot samples were amplified with P8/P2 primes. All samples were run on agarose gel and visualized under UV light. The representatives of male and female samples for each species are presented in Figure 1. The sizes of the *CHD*1-Z and *CHD*1-W PCR products were estimated in the ranges of 300−400 bp (Fig. 1). For male samples, there was a single band observed on the agarose gel. For female samples, there were double bands with different lengths on the agarose gel (Fig. 1). Of which, shorter *CHD*1 fragment was located on Z chromosome and longer *CHD*1 fragment was located on W chromosome. In most species, there were apparent differences in sizes between double gel bands of female samples. However, in *Ara ararauna*, differences in sizes of *CHD*1 genes on Z and W chromosomes were comparatively hard to examine (Fig. 1).

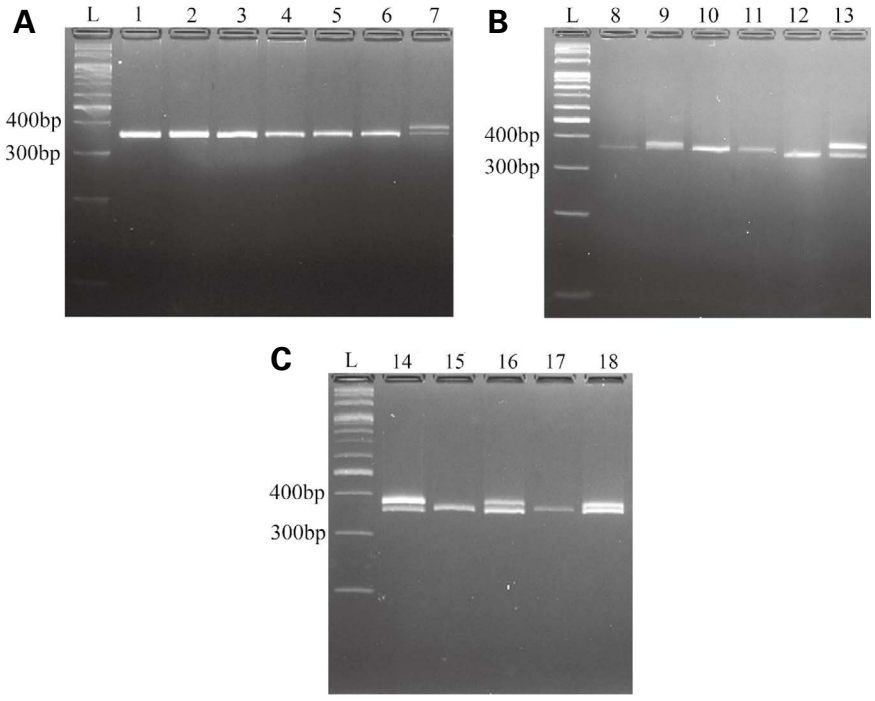

Fig. 1. PCR products visualized on an agarose gel to determine the gender of the parrots examined. L: 100 bp ladder; A. Cacatuidae: 1. *Cacatua alba* (M), 2. *C. ducorpsii* (M), 3. *C. galerita* (M), 4. *C. goffiniana* (M), 5. *C. moluccensis* (M), 6. *Nymphicus hollandicus* (M), 7. *N. hollandicus* (F). B. Psittacidae: 8. *Ara ararauna* (M,) 9. *A. ararauna* (F), 10. *A. chloropterus* (M), 11. *A. macao* (M), 12. *Myiopsitta monachus* (M), 13. *M. monachus* (F). C. Psittaculidae: 14. *Eclectus roratus* (F), 15. *Lorius garrulus* (M), 16. *L. garrulus* (F), 17. *Melopsittacus undulates* (M), and 18. *M. undulates* (F). M: male, F: female.

Based on the visualization of PCR products of collected samples on an agarose gel, the gender of parrots was identified (Table 1). There were 27 samples of 13 parrot species determined through this study (Table 1). *Cacatua alba, C. ducorpsii, C. galerita, C. goffiniana,* and *Ara macao* had only male samples, while *Eclectus roratus* had only female samples. The remaining species included both male and female samples.

The sizes of *CHD*1 gene fragments of *M. monachus* were 323 bp in Z chromosome for both male and female and 361 bp in W chromosome. Blast results indicated that *CHD*1-Z and *CHD*1-W fragment were 99.38% and 99.17% similar to *CHD*1-Z and *CHD*1-W sequence of *M. monachus* on Genbank, respective. The finding demonstrated that *CHD*1 gene was successfully amplified and its size was different between Z and W chromosomes.

Molecular based sexing has gained the popularity for gender identification in birds (Çakmak *et al.* 2017). As attractive and common birds in the pet market, parrots were targeted for gender determination in different studies. Together with other birds, the gender of various parrots were determined based on the molecular sexing technique (Griffits *et al.* 1998; Miyaki *et al.* 1998; Jensen *et al.* 2003; Vucicevic *et al.* 2013). To determine the gender of parrot from Korean zoos, this study applied P8/P2 primer set to amplify partial *CHD*1 gene, and PCR products were screened by gel electrophoresis. With this method, all collected parrots were determined gender. Our finding is congruent with previous studies on bird sexing based on P8/P2 primers (Griffits *et al.* 1998; Jensen *et al.* 2003). P8/P2 primers were designed to bind two exons and flank an intron (Griffits *et al.* 1998). The size of PCR products amplified with P8/P2 varied from 300−400 bp and showed the difference between Z and W chromosomes (Griffits *et al.* 1998). The differences in the sizes of *CHD*1-Z and *CHD*1-W fragments resulted in a single (*CHD*1-Z) band in male parrots and double bands (*CHD*1-Z and *CHD*1-W) in female parrots. Two bands in female parrots indicated the presence of a shorter *CHD*1 gene product of Z chromosome and a longer *CHD*1 gene of W chromosome (Griffits *et al.* 1998; Miyaki *et al.* 1998; Jensen *et al.* 2003). Therefore, amplification of *CHD*1 gene with P8/P2 primers is able to discriminate parrot gender.

In *A. ararauna* (Fig. 1b), the separation between *CHD*1-Z

Table 1. Parrot species and their gender determined in this study

Family	Species name	Common name	Number of sample	Form of sample	Zoos	Gender ratio (M/F)*
Cacatuidae	*Cacatua alba*	White cockatoo	1	Blood	Seoul zoo	1/0
	Cacatua ducorpsii	Solomons cockatoo	1	Feather	Uchi zoo	1/0
	Cacatua galerita	Sulphur-crested cockatoo	1	Blood	Seoul zoo	1/0
	Cacatua goffiniana	Tanimbar corella	1	Blood	Seoul zoo	1/0
	Cacaua moluccensis	Salmon-crested cockatoo	1	Feather	Seoul zoo	1/0
			1	Feather	Cheongju Zoo	1/0
	Nymphicus hollandicus	Cockatiel	2	Feathers	Seoul zoo	1/1
			2	Feathers	Cheongju Zoo	1/1
Psittacidae	*Ara ararauna*	Blue-and-yellow macaw	1	Feather	Seoul zoo	1/0
			1	Feather	Cheongju Zoo	1/0
			1	Feather	Uchi zoo	0/1
	Ara chloropterus	Red-and-green macaw	1	Feather	Seoul zoo	1/0
			1	Feather	Uchi zoo	1/0
	Ara macao	Scarlet macaw	1	Feather	Seoul zoo	1/0
	Myiopsitta monachus	Monk parakeet	2	Feathers	Seoul zoo	1/1
Psittaculidae	*Eclectus roratus*	Eclectus parrot	1	Feather	Seoul zoo	0/1
			1	Feather	Uchi zoo	0/1
	Lorius garrulus	Chattering lory	1	Feather	Seoul zoo	0/1
			1	Feather	Cheongju Zoo	1/0
	Melopsittacus undulatus	Budgerigar	2	Feathers	Seoul zoo	1/1
			2	Feathers	Cheongju Zoo	1/1
			1	Feather	Uchi zoo	0/1

*M: male; F: female

band and *CHD*1-W band was not clear. In the previous study, Vucicevic *et al.* (2013) were unable to identify gender for this species with P8/P2 primers, stating that there was little distance between *CHD*1-Z and *CHD*1-W in PCR products. In this study, even though *CHD*1-Z band and *CHD*1-W band were close, female individuals of *A. ararauna* can still be predicted, especially when their gel bands were compared to a thin band of male individual of the same species.

In conclusion, this study determined gender for parrots collected from Korean zoos. The finding confirmed that the analysis of *CHD*1 sequence based on PCR technique is valuable for gender determination in parrots. These results are helpful for management and caring of parrots in Korean zoos. Due to limitation of samples in the zoos, we could not analyze both male and female for all species. Future studies are recommended to apply molecular sexing to both genders for each species, which is important for research and conservation programs of rare and endangered birds.

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REFERENCES

Bush ER, SE Baker and DW Macdonald. 2014. Global trade in exotic pets 2006–2012. Conserv. Biol. 28:663–676.

Çakmak E, AP Çigdem and BC Can. 2017. Comparison of three different primer sets for sexing birds. J. Vet. Diagn. Invest. 29:59–63.

Ellegren H. 1996. First gene on the avian W chromosome (*CHD*) provides a tag for universal sexing of non-ratite birds. Proc. R. Soc. Lond. B-Biol. Sci. 263:1635–1644.

Griffiths R, MC Double, K Orr and RJ Dawson. 1998. A DNA test to sex most birds. Mol. Ecol. 7:1071–1075.

Griffiths R and B Tiwari. 1995. Sex of the last wild Spix's macaw. Nature 375:454.

Ito H, A Sudo-Yamaji, M Abe, T Murase and T Tsubota. 2003. Sex identification by alternative polymerase chain reaction methods in Falconiformes. Zool. Sci. 20:339–344.

Jensen T, FM Pernasetti and B Durrant. 2003. Conditions for rapid sex determination in 47 avian species by PCR of genomic DNA from blood, shell-membrane blood vessels, and feathers. Zoo Biol. 22:561–571.

Kahn NW, J St John and TW Quinn. 1998. Chromosome-specific intron size differences in the avian CHD gene provide an efficient method for sex dentification in birds. The Auk 115:1074–1078.

Lee J, L Tsai, P Hwa, C Chan, A Huang, S Chin, L Wang, J Lin, A Linacre and H Hsieh. 2010. A novel strategy for avian species and gender identification using the CHD gene. Mol. Cell. Probes 24:27–31.

Miyaki CY, R Griffiths, K Orr, LA Nahum, SL Pereira and A Wajntal. 1998. Sex identification of parrots, toucans, and curassows by PCR: perspectives for wild and captive population studies. Zoo Biol. 17:415–423.

Valadan R, F Nejatollahi, H Ehsani-Nori, H Habibi, H Amini and M Aliabadian. 2017. Avian gametologs as molecular tags for sex identification in birds of prey of Iran. Zoo Biol. 36:289–293.

Vucicevic M, M Stevanov-Pavlovic, J Stevanovic, J Bosnjak, B Gajic, N Aleksic and Z Stanimirovic. 2013. Sex determination in 58 bird species and evaluation of CHD gene as a universal molecular marker in bird sexing. Zoo Biol. 32:269–276.

